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The Epstein-Barr Virus Nuclear Protein 1 Promoter Active in Type I Latency Is Autoregulated

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The only member of the Epstein-Barr virus family of nuclear proteins (EBNAs) expressed during type I and type II latent infections is EBNA-1. This is in contrast to type III latency, during which all six nuclear proteins are expressed from a common transcription unit. The exclusive expression of EBNA-1 during type I and II latency is mediated through a recently identified promoter, Fp. The objective of this study was to characterize Fp in the Burkitt lymphoma cell background, where it is known to be differentially utilized. Using a short-term transfection assay and reporter gene plasmids containing Fp linked to the human growth hormone, we examined Fp activity in type I and type III latently infected and virus-negative Burkitt lymphoma cells. The data suggested that Fp is predominantly regulated through two distinct elements located between +24 and +270 relative to the transcription start site. One element positively mediates Fp activity, probably at the level of transcription, and acts in a virus-independent manner. The second element contains the EBNA-1 DNA binding domain III and negatively regulates Fp-directed gene expression in *trans* with EBNA-1 in type III as well as type I latency. Thus, we have identified a third function of EBNA-1, i.e., that of a repressor of gene expression, in addition to its known role in viral DNA replication and its ability to *trans*-activate gene expression. The overall activity of Fp in type I latently infected Burkitt cells was approximately sixfold lower than in virus-negative Burkitt cells, in which there is no autoregulation, suggesting that there is a fine balance between these two opposing regulatory elements during type I latency.

Epstein-Barr virus (EBV) has the ability to establish a latent infection and is maintained in a life-long carrier state, a hallmark of all herpesviruses. Historically, the in vitro model of EBV latency has been the EBV-immortalized or growth-transformed B lymphoblastoid cell line (LCL) and long-term cultures of Burkitt lymphoma (BL) cells. Latent infection within these cells is characterized by the expression of 11 viral genes, 9 of which encode the latent-infection proteins: the six Epstein-Barr nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, and leader protein) and the three latent membrane proteins (LMPs 1, 2A, and 2B). The remaining two genes encode the Epstein-Barr virus-encoded RNAs (EBERs), which are small RNAs that bear resemblance to the mammalian small nuclear ribonucleoproteins, though their function is unknown. The expression of these viral genes in continuous B-cell lines, Burkitt tumor cells in vitro, and posttransplant lymphomas, together with many of the known biochemical properties of the latent-infection proteins, strongly supports their role in the maintenance of latency and/or the cell transformation process (see references 21 and 31 for a review).

More recent analyses of EBV latent-gene expression in cells from actual BL and nasopharyngeal carcinoma (NPC) biopsy samples have revealed alternative states of latency characterized by more restrictive patterns of viral gene expression. In BL tumors, the only EBV gene products known to be expressed are the EBNA-1 protein and EBERs (9, 15, 28, 29, 30). This pattern of latent-gene expression or state of latency, referred to as the group I phenotype or type I latency, respectively, is generally not stable in vitro, and upon continued passage, BL cells convert to the group III or LCL-like pattern of viral gene expression (type III latency [15, 29]). Within NPC tumors and NPC cells that have been passaged in nude mice, there is expression of the latent membrane proteins in addition to EBNA-1 and the EBERs (10–12, 18, 22, 36, 46). This pattern of EBV gene expression, type II latency, has recently been described in BL cells also (29). Moreover, a novel set of overlapping RNA transcripts are characteristically expressed in NPC cells and may further differentiate EBV latency in epithelial cells from latency in B lymphocytes (11, 18).

Although type III latency must occur in otherwise healthy individuals, as evidenced by humoral and cellular immune responses to most of the latent-infection proteins, it is less certain whether type I and II latency also occur in healthy persistently infected individuals, since these states of infection have been observed only in tumor cells. Clearly, a major advantage of maintaining a less active state of latency, in tumor cells as well as in nontransformed cells, would be the ability to evade the immune surveillance of the host. In fact, the phenomenon of type I latency was first described as the resistance of BL cells to human leukocyte antigen-restricted T-cell killing in vitro (14, 27). Interestingly, attempts to demonstrate a cellular immune response to EBNA-1 have to date been unsuccessful, and thus, B cells that maintain a type I latency may not be perceived as EBV infected. Because EBNA-1 is required in *trans* for replication of the EBV genome in latently infected cells (44, 45), its expression would be critical in a population of naturally proliferative B cells to ensure the passage of EBV genomes to progeny B cells and, presumably, the maintenance of a persistent infection.

An intriguing aspect of multiple states of EBV latency is the differential expression of the latent-infection proteins, particularly the EBNA proteins. In type III latency, the EBNA mRNAs are encoded by the same transcription unit that is under the control of one of two alternatively used

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promoters, Wp and Cp (6, 33, 40, 41). Through transcriptional and posttranscriptional mechanisms that currently are poorly understood, the precursor RNAs are differentially processed to yield the individual EBNA mRNAs (5, 34). Comparative analysis of the steady-state levels of the latent-infection gene mRNAs in type I and type III subclones of an African BL suggests that the latent-gene promoters active during type III latency are silent during type I latency (32). We and others have shown that the dissociation of EBNA-1 expression from the other EBNA proteins in type I BL cells is mediated through the activation of a previously unknown promoter, Fp, that is used for the exclusive expression of EBNA-1 (32, 35). Expression of EBNA-1 during type II latency in NPC cells is also mediated through the Fp promoter (18, 37). Thus, the differential expression of the latent-infection genes appears to be primarily mediated at the level of transcription.

A thorough knowledge of the mechanisms that regulate the expression of the latent-infection proteins through previously identified transcriptional promoters is of course paramount to understanding the ability of EBV to establish and maintain a latent infection. Equally important to this process is the regulation of EBNA-1 expression through the Fp promoter, particularly since the more restricted patterns of gene expression exhibited during type I and II states of latency are likely avenues through which the virus persists in an immunocompetent host. In this communication, we present evidence that Fp contains two major regulatory elements, both of which are located downstream of the transcription start site. One of these positively mediates Fp activity in an EBV-independent manner. The second element contains two EBNA-1 binding sites and acts in *trans* with EBNA-1 to negatively regulate Fp activity. Thus, in addition to its previously identified roles in DNA replication and transactivation of gene expression (25, 39, 45), EBNA-1 can also repress gene expression.

MATERIALS AND METHODS

Cell lines. All cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. The Mutu BL cell lines employed in these studies maintained either a type I (clone 59) or a type III (clone 176) latent EBV infection, as routinely determined by immunoblot analysis of EBNA expression. The derivation of these clonal BL cell lines from a biopsy of an African BL has been described elsewhere (15). Louckes cells are an EBV-negative BL cell line.

Construction of reporter gene plasmids. The gene for human growth hormone (hGH) was used as a reporter of Fp activity in transient-expression assays. Restriction fragments of EBV DNA spanning the Fp promoter region were inserted by blunt-end ligation into the *Bam*HI site immediately upstream of a promoterless hGH gene in the plasmid pφGH (Nichols Institute, San Juan Capistrano, Calif.). Two sets of constructs were made and are illustrated in Fig. 1. In the first set, the 3′ boundary of all of the EBV DNA inserts was the *Bam*HI restriction site at position +24 relative to the transcription start site; the 5′ boundaries of these extended to a *Mlu*I (−732), *Bgl*I (−488), or *Xma*III (−367) restriction site. The second set of constructs had the same 5′ boundaries but extended to the *Pvu*II restriction site at +270 and contained EBNA-1 DNA binding region III (3, 20, 24). A 34-bp deletion that precisely spans this EBNA-1 binding domain was introduced into the plasmid pφGH.006Δ34 by the method of recombinant polymerase chain reaction described by Higuchi (17). All reporter gene plasmids were purified by two cycles of cesium chloride-ethidium bromide gradient centrifugation. Where indicated, Fp-hGH reporter plasmids were methylated in vitro with *Hpa*II or CpG methylase (New England Biolabs) and *S*-adenosylmethionine. Mock methylation of plasmid DNA was done under identical conditions except that methylase was omitted from the reaction. Prior to transfection with these plasmids, the inability of *Hpa*II endonuclease to digest methylated relative to mock-methylated DNA was confirmed to ensure that the methylation reactions had been successful.

DNA transfections and growth hormone assay. For each transfection, 8×10^6 cells were suspended in 0.25 ml of fresh growth medium, mixed with 15 μg (unless indicated otherwise) of reporter plasmid DNA in a 0.4-cm electrode gap cuvette (Bio-Rad), and allowed to stand at room temperature for 5 min. Transfection was achieved by electroporation using a Bio-Rad Gene Pulser apparatus set at 250 V and a capacitance of 960 μF. After standing on ice for 10 min following electroporation, the cells were removed from the cuvettes by rinsing them with 0.7 ml of ice-cold phosphate-buffered saline and placed into 11 ml of growth medium that consisted of 1 ml of conditioned medium and 10 ml of fresh medium containing antibiotics. Fp activity was determined by measuring the amount of hGH present in the growth medium at 40 h posttransfection with a radioimmunoassay system (Nichols Institute) according to the manufacturer's instructions. All transfections and hGH determinations were done in duplicate. To factor out variation in hGH determinations due to differences in the ages and lots of assay reagents, we converted counts per minute to relative promoter activity units. This was done by dividing the counts per minute (amount of hGH produced) from a test construct by the baseline amount of hGH detected in the culture medium of cells transfected with the promoterless hGH plasmid pφGH. Baseline expression was consistent and typically was 100 cpm above background. However, this was due to the diluent (fetal bovine serum) and not to expression from pφGH. Actual counts per minute can be deduced by multiplying relative promoter activity units by 100 to 150 cpm. To ensure that measured differences in Fp activity were not due to variations in transfection efficiency, we routinely cotransfected our reporter plasmids with 1 μg of pSV_2CAT, which contains the bacterial chloramphenicol acetyltransferase gene under transcriptional control of the simian virus 40 early promoter and enhancer element (13); chloramphenicol acetyltransferase activity in transfected-cell extracts was then determined (13) to monitor for significant differences in transfection efficiency. In several experiments, Fp-hGH reporter plasmids were cotransfected with an EBNA-1 expression plasmid, pSV.E1, in which EBNA-1 expression is under the control of the simian virus 40 early promoter. pSV.E1 was constructed by inserting a *Sau*3A-*Pvu*II subfragment (genomic coordinates 107930 to 110174 [4]) of the EBV *Bam*HI-K restriction fragment into the *Bam*HI site of the eukaryotic expression vector pSG5 (Stratagene).

Analysis of RNA. Total cell RNA was isolated by acid phenol-guanidinium thiocyanate extraction (8) using RNAzol B (Biotecx Laboratories, Houston, Tex.) according to the manufacturer's instructions except that an additional phenol-chloroform and a chloroform extraction were performed after the initial extraction with RNAzol B. Prior to Northern (RNA) blot analysis, RNA was treated with RQ1 DNase (Promega) to remove contaminating DNA. Ten micrograms of RNA per lane was fractionated in a 1.2% agarose–2.2 M formaldehyde gel, transferred to a Gene-

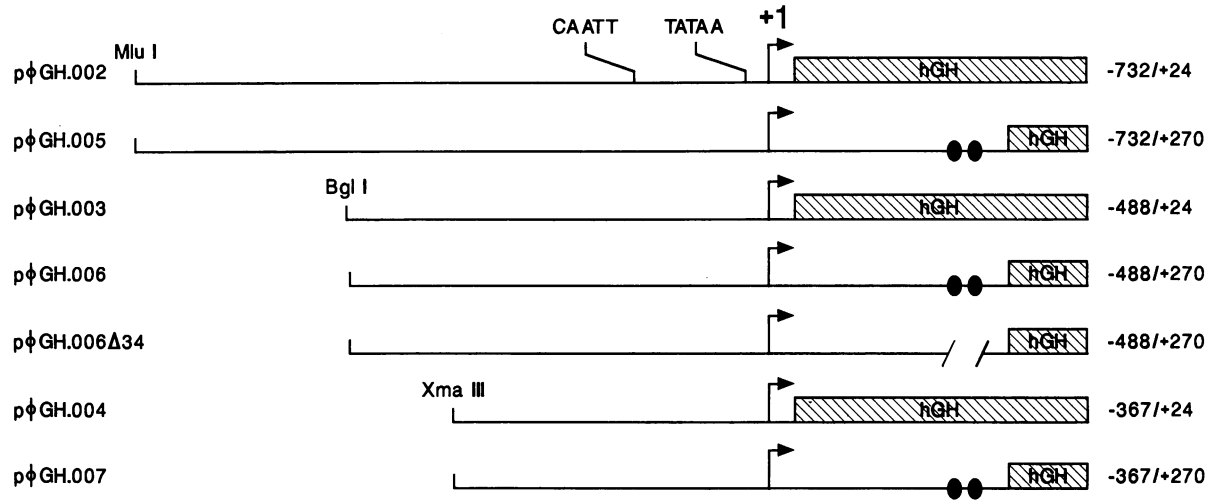

FIG. 1. Fp-hGH reporter gene plasmids. Plasmid designations are listed to the left, and EBV DNA coordinates relative to the transcription start site (bent arrow) are listed to the right of the schematic depiction of each construct. Positions +24 and +270 are the last bases of the EBV *Bam*HI and *Pvu*II restriction sites, respectively, that are present in the constructs. The genomic coordinates of restriction sites are as follows: *Mlu*I, 61500; *Bgl*I, 61744; *Xma*III, 61866; *Bam*HI, 62249; *Pvu*II, 62499 (4). The two EBNA-1 binding sites within each region III DNA binding domain (3, 20) are represented by the black ellipses.

Screen Plus (NEN/DuPont) nylon membrane, and hybridized to a ^{32}P-labeled RNA probe complementary to the first exon of the hGH mRNA. Prehybridization and hybridization of RNA blots were carried out for 2 and 15 h, respectively, at 65°C in 50% formamide–5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–50 mM Tris-HCl (pH 7.5)–0.1% PP_i–1% sodium dodecyl sulfate–0.2% each of polyvinylpyrrolidone, Ficoll, and bovine serum albumin–5 mM EDTA. Following hybridization, blots were washed as described elsewhere (34) and exposed to Kodak XAR-5 film at −70°C.

RESULTS

Major regulatory elements of Fp downstream of the site of transcription initiation. Our first objective was to identify the *cis* elements of Fp that are required for Fp-directed gene expression and evaluate these within the context of various BL cell lines in which Fp is or is not active. This was done by employing a reporter gene assay in which EBV DNA fragments spanning the Fp promoter region were inserted upstream of a promoterless hGH gene. Three sets of constructs were made such that the boundaries of the EBV DNA extended 5′ of the transcription start site to −732, −488, or −367 and 3′ to either +24 or +270 (Fig. 1). Those that extended to +270 encompassed EBNA-1 DNA binding domain III (3, 20, 24), which contains two EBNA-1 binding sites located between +203 and +236, and were constructed to examine the possible role of EBNA-1 in regulating expression from Fp. Fp-directed expression of hGH was determined following transfection of two subclonal cell lines of the Mutu BL (15): one that exhibited a type I EBV latency (clone 59) and one that exhibited a type III latency (clone 176). Because these Mutu BL cell lines are isogenic, our results should not have reflected any differences due to genetic background or between endogenous virus. We were surprised to find that the relative promoter activity of Fp within our reporter plasmids was very low in the type I BL cells (Fig. 2A), even though the endogenous promoter is utilized for EBNA-1 expression in these cells (32). We did note, however, that in type I BL cells, the levels of hGH produced from cells that were transfected with the constructs extending to +270 were consistently higher, though not remarkably so, than the hGH levels produced from the constructs that extended to +24. Virtually identical results

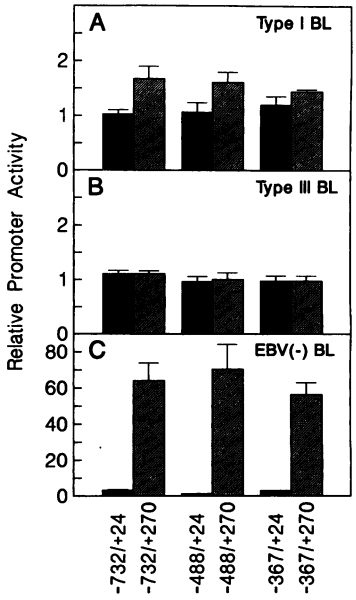

FIG. 2. Fp promoter activity in BL cells. Fp-directed expression of hGH was used as an indicator of promoter activity in type I (A) and type III (B) latently infected Mutu BL cells and in EBV-negative Louckes BL cells (C) following transfection with reporter plasmids as described in the legend to Fig. 1. The 5′ and 3′ coordinates of the EBV DNA in each plasmid are presented at the bottom. Constructs extending to position +24 or +270 are represented by solid and hatched columns, respectively. Relative promoter activity was determined as described in Materials and Methods. Data are the means of two representative experiments (four transfections with each construct), and bars indicate the standard errors of the means.

were obtained with the Akata BL cell line, which also maintains a type I latency (data not shown). We observed no significant level of Fp activity in the type III BL cells (Fig. 2B), suggesting that Fp may be specifically down-regulated during type III latency, as one might expect in a cell line in which the endogenous promoter is inactive. However, when we employed the IB4 LCL as a model of type III latency, the constructs that extended 3′ to +270 yielded greater Fp activity than those extending only to +24 (data not shown), similar to the results obtained with the type I BL cells. Although the difference between results obtained with type III BL cells versus an LCL may reflect a difference between transformed and nontransformed cells, respectively, we feel that it is more likely due to the poor transfection efficiency of the type III BL cells coupled with the inherently low activity of Fp in EBV-infected cells.

When we transfected these same constructs into the EBV-negative BL cell line Louckes, the levels of hGH produced from the constructs extending to +270 were approximately 25-fold higher than those produced from the constructs extending only to +24 (Fig. 2C), which, as in the type I and III BL cells, were at or only slightly above baseline expression. We also observed no noticeable differences between Fp activity in constructs that extended to +270 but contained different lengths of EBV DNA upstream of the transcription start site. The same relative observations were made when experiments were repeated with the EBV-negative B-cell lymphoma line BJAB (data not shown). Since we observed only minimal activity of Fp from reporter plasmids containing various lengths of upstream sequence but lacking information derived from DNA between +24 and +270, we concluded that Fp activity is predominantly mediated through an element(s) located between +24 and +270. When we took into consideration the approximately fivefold-lower transfection efficiency of our type I BL cells relative to the EBV-negative BL cells, the activity of Fp in the type I BL cells was estimated to be sixfold lower than the same constructs (those extending to +270) in the EBV-negative cells. This suggested that significant down-regulation of Fp was occurring in type I BL cells.

EBNA-1 negatively affects Fp-directed gene expression. The apparent down-regulation of Fp activity that occurred when reporter plasmids containing the EBNA-1 binding sites were introduced into type I BL cells, in which the only viral protein known to be expressed is EBNA-1, suggested that EBNA-1 may negatively regulate its expression through Fp. We tested this hypothesis by cotransfecting EBV-negative BL cells with the Fp-hGH reporter plasmid pϕGH.006 (−488/+270) and the EBNA-1 expression vector pSV.E1. EBNA-1 repressed Fp-directed expression of hGH in a dose-dependent manner in these cells relative to cells that were transfected with pϕGH.006 and the expression vector without the EBNA-1 coding sequence, pSG5 (Fig. 3A). To confirm that this repression was actually mediated through the previously identified EBNA-1 DNA binding sites, EBV-negative BL cells were transfected with pϕGH.006Δ34, which lacks the EBNA-1 binding sites, or were cotransfected with pϕGH.006Δ34 and either pSG5 or pSV.E1. Whereas hGH expression from pϕGH.006, which contained the EBNA-1 binding sites, was inhibited by 85% when the plasmid was cotransfected with 0.5 μg of pSV.E1 (data not shown and Fig. 3A), no significant inhibition of hGH expression from pϕGH.006Δ34 was observed when this plasmid was cotransfected with pSV.E1 (Fig. 3B). Thus, negative regulation of Fp-directed gene expression by EBNA-1 is mediated through these binding sites. Furthermore, since

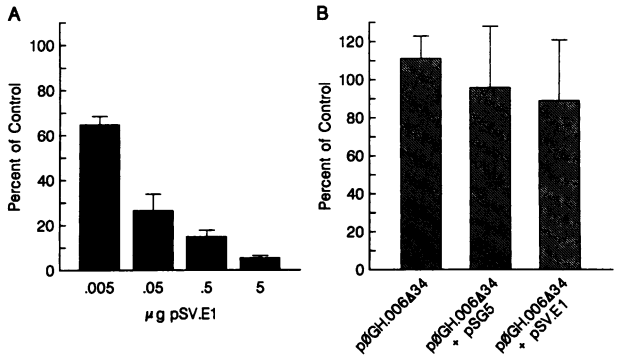

FIG. 3. Inhibition of Fp-directed gene expression by EBNA-1. (A) Louckes BL cells were transfected with 15 μg of pϕGH.006 (−488/+270) and the indicated amount of the EBNA-1 expression plasmid pSV.E1. The levels of hGH (Fp activity) produced by these cells are expressed as the percentage of hGH that was produced from cells cotransfected with pϕGH.006 and the same relative amount of pSG5 instead of pSV.E1. (B) Louckes cells were transfected with 15 μg of the indicated reporter gene plasmid alone or cotransfected with 0.5 μg of either pSG5 or pSV.E1. Percentage of control was calculated relative to the mean level of hGH expressed from cells transfected with pϕGH.006. All data points in this figure represent the means of at least two experiments, and bars indicate standard errors. In the absence of EBNA-1, Fp activity in pϕGH.006 was equivalent to values reported in Fig. 2C.

pϕGH.006 and pϕGH.006Δ34 were equally active, the downstream positive regulatory element of Fp must be distinct from the EBNA-1 binding domain, and because these cells were EBV negative, this element must be regulated in a virus-independent manner.

The results that we obtained from our analysis of Fp activity by hGH assay were also reflected in mRNA levels in that transcripts were detectable only in those cells that had been transfected with an Fp-hGH construct that contained the downstream sequence from +24 to +270 and only in the absence of EBNA-1 (Fig. 4, pϕGH.006). The fact that we did not detect any hGH transcripts within the cells that received a reporter plasmid that did not contain the downstream positive regulatory element (Fig. 4, pϕGH.003) suggests that this element transcriptionally regulates Fp. We cannot, however, make the same assumption about the mechanism of EBNA-1-mediated regulation of Fp function based on these data. In fact, since the region III EBNA-1 binding domain is also present within the RNA transcript, it is possible that EBNA-1 acts posttranscriptionally through an RNA-protein interaction to negatively affect mRNA stability, transport, or processing.

EBNA-1 is a major determinant of Fp activity during type I and III latency. The data that are presented in Fig. 2A indicated that Fp activity was low, at least within our reporter plasmids, in type I latently infected BL cells. These results may indicate that there is a net balance between the opposing regulatory elements we have identified that results in a relatively low overall activity of Fp, although it is also possible that our reporter gene constructs did not contain an element(s) that may counter the negative affect of EBNA-1 during type I latency. To determine whether EBNA-1 was down-regulating apparent Fp activity in type I latency, we examined the relative levels of hGH expressed from the pϕGH.006 and pϕGH.006Δ34 reporter plasmids following transfection of type I BL cells (Fig. 5). The data indicated that removal of the EBNA-1 binding sites resulted in a

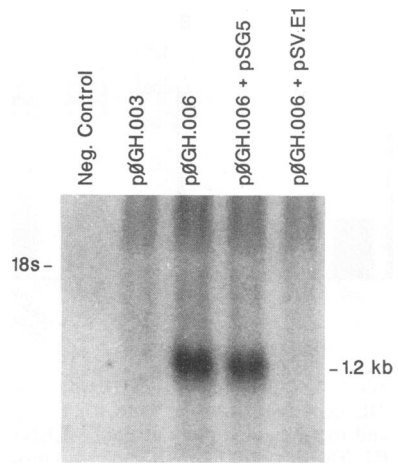

FIG. 4. Steady-state hGH mRNA levels in transfected EBV-negative BL cells. A Northern blot containing 10 μg of total cell RNA from untransfected (Neg. Control) or transfected Loukes cells per lane was probed with a ^{32}P-labeled cRNA of the first exon of hGH. Cells were transfected with 19 μg of reporter plasmid alone or cotransfected with 1 μg of either pSG5 or pSV.E1. With the exception of a lower amount of RNA present in the negative control lane, all lanes contained an equivalent amount of RNA as determined by rRNA content (not shown). The position to which the 18S rRNA migrated is indicated. The expected sizes of EBV hGH mRNAs in cells transfected with pφGH.003 or pφGH.006 are 0.9 and 1.2 kb, respectively.

fivefold increase in relative promoter activity. Furthermore, the relative promoter activity of Fp within pφGH.006Δ34 was equivalent to the activity of the cytomegalovirus immediate-early enhancer–human T-cell lymphotropic virus type I (HTLV-I) long terminal repeat cassette, a very active promoter in our cell lines. These data suggest, therefore, that

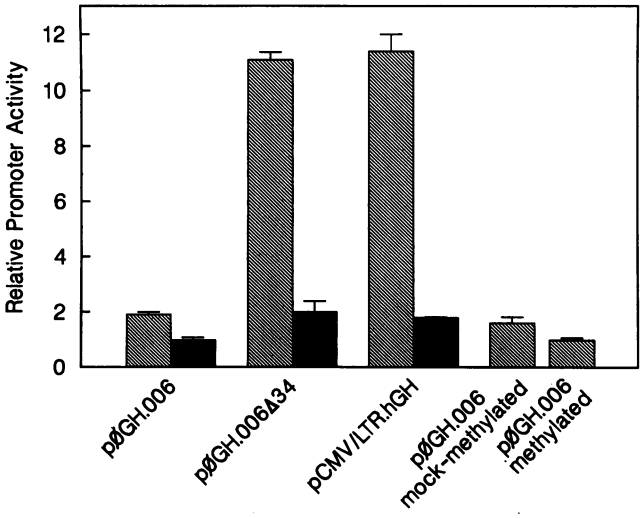

FIG. 5. EBNA-1 is a major negative regulator of Fp activity in type I and type III latently infected BL cells. Type I (▨) and type III (■) latently infected Mutu BL cells were transfected with the indicated Fp-hGH reporter plasmid that contained the EBNA-1 DNA binding domain III (pφGH.006) or that had it deleted (pφGH.006Δ34). pCMV/LTR.hGH (see text) was used as a positive control for promoter activity. Data represent the means of two representative experiments, and bars indicate standard errors.

TABLE 1. Mutation of splice donor within second EBNA-1 binding site does not disrupt EBNA-1-mediated repression

Reporter gene plasmid[a]	BL cell type[b]	
	Type I	EBV (−)
pφGH.006	6.5 (±1.1)	ND
pφGH.006Δ34	41.3 (±12.1)	ND
pφGH.016[c]	3.6 (±0.8)	ND
pφGH.006 + pSG5	ND	153.6 (±6.2)
pφGH.006 + pSV.E1	ND	18.2 (±2.6)
pφGH.016 + pSG5	ND	76.7 (±23.0)
pφGH.016 + pSV.E1	ND	8.8 (±2.8)

[a] All transfections were done with 15 μg of hGH reporter plasmid alone or with 1 μg of either pSG5 or pSV.E1.

[b] Values are the means of relative promoter activity (± standard deviation) from two experiments done in duplicate. ND, not done.

[c] pφGH.016 is identical to pφGH.006 (−488/+270) except that the G at position 4 of the second EBNA-1 binding site (CGG/GTAATAC) has been mutated to an A.

EBNA-1 is a major determinant of overall Fp activity during type I latency. Although the activity of Fp within these same constructs was lower in type III cells, largely because of transfection efficiency, the same relative results were obtained (Fig. 5), suggesting that EBNA-1 may be a major factor in preventing expression from Fp during type III latency. A potential complication of our interpretation, however, is that in deleting the EBNA-1 binding sites, we have also removed a splice donor site located between positions 3 and 4 of the second binding site (CGG/GTAATAC). The loss of this site alone could result in an increase in hGH production, especially if EBNA-1 mediates the use of this splice site. Therefore, we converted the G at position 4 to an A by recombinant polymerase chain reaction (17). This change should be allowable according to a previous study of EBNA-1 binding affinities for different sites (3) and should also inactivate the splice donor, since the GT dinucleotide is virtually invariant among mammalian splice donor sites. The resulting plasmid, pφGH.016, was then evaluated in type I BL and EBV-negative BL cells (Table 1). The data indicated, however, that mutation of the splice donor site did not result in an increase in hGH expression in the type I BL cells. In fact, Fp activity was actually decreased, probably because of an increase in the binding affinity of EBNA-1 for the mutated site. Furthermore, EBNA-1 was still capable of repressing hGH expression from pφGH.016 in cotransfection experiments in the EBV-negative Louckes BL cells.

Because the EBV genome in general is hypermethylated in type I latently infected cells relative to type III latency (23), it occurred to us that methylation of key CpG elements within Fp may disrupt negative regulation, e.g., by preventing the interaction of EBNA-1 with its binding sites. Thus, full activation of Fp during type I latency may require that the promoter be methylated to some degree. Examination of the methylation status of genomic DNA spanning the Fp promoter region with several methylation-sensitive restriction endonucleases indicated that this region is differentially methylated during type I latency relative to type III latency, where it is essentially unmethylated (31a). With the exception of two CpG pairs within *Hpa*II sites present in our constructs, one of which is located between the two EBNA-1 binding sites (there are five CpG pairs within the region III binding domain), CpG pairs within the six other *Hpa*II recognition sites represented in our constructs all

appeared to be methylated. Therefore, to best mimic the differential methylation pattern of Fp in its transcriptionally active state, we methylated pφGH.006 with *Hpa*II methylase in vitro prior to transfection into type I BL cells. Relative to mock-methylated pφGH.006, however, we observed no increase in Fp activity as a result of methylation (Fig. 5). Identical results (not shown) were obtained with pφGH.006 methylated with CpG methylase, a prokaryotic methylase that methylates all CpG pairs. However, total methylation of pφGH.006 abolished Fp activity in our EBV-negative BL cells. Thus, since we were unable to precisely mimic the differential methylation of Fp that occurs in vivo and since total methylation abolished Fp activity in the absence of EBNA-1, we could not conclude whether DNA methylation interferes with negative regulation of Fp by EBNA-1.

DISCUSSION

We have shown that Fp, the promoter used for the exclusive expression of EBNA-1 in type I latent infection of BL cells, contains two major regulatory elements. Unlike classical *cis* regulatory elements, which are commonly, though not always, located 5′ of the transcription start site, both of these regulatory elements of Fp are located downstream of the transcription start site. Moreover, these elements are functionally opposite in their effects on Fp-directed gene expression. Although we are currently working toward identifying and characterizing the positive regulatory element, we have shown here that negative regulation of Fp is mediated by EBNA-1 in *trans* with the second element, the EBNA-1 DNA binding domain III previously identified by Rawlins, Hayward, and colleagues (3, 20, 24). Thus, this is the first indication of the function of the region III binding domain as well as the demonstration of a third function of EBNA-1, that of a repressor of gene expression, in addition to its previously known role in viral DNA replication and a proposed role in the transactivation of the Cp EBNA promoter during type III latency (25, 39, 45).

We found Fp activity, as determined by our reporter gene assay, to be low in type I and III latent infections of BL cells. In type I BL cells, this was due predominantly, if not completely, to EBNA-1, since constructs in which the EBNA-1 binding sites had been deleted appeared to be fully active or at least as active as our positive control promoter (Fig. 5). We made the same relative observation in type III BL cells, suggesting that all of the factors necessary for the activation of Fp are present during type III latency and that, therefore, EBNA-1 that is expressed from the transcriptionally active Cp and Wp promoters could effectively prevent expression from Fp during type III latency. However, because reporter gene constructs that contained both regulatory elements were virtually inactive in type III relative to type I BL cells (Fig. 1) and because Fp activity in the construct lacking the EBNA-1 binding sites was only twofold higher than the construct containing the binding sites (Fig. 5), we cannot exclude the possibility of an additional level of regulation during type III latency. Although we could largely attribute low levels of Fp activity in these cells to the fivefold-lower transfection efficiency of our type III relative to type I BL cells, we are currently examining Fp activity in other type III BL cells. Because Fp is naturally within the context of the viral genome, expression from this promoter during type III latency may be additionally restricted by transcriptional interference from the Cp and Wp EBNA promoters.

EBNA-1 levels in type I BL cells do not appear to be significantly different from levels present within type III BL cells or LCLs as analyzed by immunoblotting (15, 28, 29, 32). In fact, Sternas et al. have determined that the actual number of EBNA-1 molecules per cell varies less than twofold among 10 different B-cell lines that they examined (38). Assuming that EBNA-1 binds as a homodimer in vivo (2) and that the number of binding sites within the genomes of different EBV isolates does not vary appreciably from the number in the prototype strain B95-8, data from this same study indicate that most cell lines contain at least a 10-fold excess of EBNA-1 relative to its binding sites (discussed below). Although these findings do not distinguish between different posttranslationally modified species of EBNA-1, which may have altered functions, it does raise the question of how EBNA-1 is produced in type I latency in the face of an apparently efficient autofeedback control mechanism. One mechanism that we were unable to completely exclude, because of our inability to precisely mimic differential methylation of Fp in vitro, is that methylation of CpG pairs within the EBNA-1 DNA binding domain III prevents the binding of EBNA-1 and therefore disrupts the negative effect that EBNA-1 has on expression. DNA methylation, however, is involved in the down-regulation of EBV latent gene expression in type I latency. This was suggested by observations that inhibition of methylation in type BL cells with 5-azacytidine resulted in the activation of the Wp-Cp EBNA promoters (35) and induced expression of the full array of latent gene proteins (23). Most recently, Jansson and colleagues have provided direct evidence that methylation of CpG sites within the enhancer element of the Wp EBNA promoter inhibits the activity of this promoter in type I latently infected BL cells (19).

It is entirely possible that the relatively low level of Fp activity we observed in type I BL cells accurately reflects the activity of the endogenous promoter in type I latency. If this is correct, we would predict that the "strength" of Fp would be largely determined by the net effect of the two opposing regulatory elements that we have identified. Assuming that basic components of the cellular transcriptional machinery and levels of any *trans*-acting factors that positively mediate Fp activity are not rate limiting, there are several possible mechanisms whereby sufficient levels of EBNA-1 could be maintained in the face of this autofeedback mechanism. Because the DNA binding affinity of EBNA-1 is lowest for the region III binding domain (3, 20), under lower levels of EBNA-1, the majority of molecules would be bound to the higher-affinity sites in binding regions I and II (3, 7, 20, 24, 26, 42) within the origin of DNA replication, *oriP*, and would be unavailable for autoregulation of Fp. As total occupancy of these sites is approached, one would expect there to be a greater interaction of EBNA-1 with the region III domain and, as a result, repression of further expression of EBNA-1. This would ensure that sufficient EBNA-1 was available to mediate DNA replication and may also explain why a greater number of EBNA-1 binding sites are maintained within *oriP* than are required for DNA replication (7, 42).

Availability of EBNA-1 could also be mediated by at least two other means. First, since EBNA-1 binds DNA as a homodimer (2), it is possible that formation of a heterodimer with a cellular protein could prevent EBNA-1 from binding. This could occur under conditions of low levels of EBNA-1 or at a given point in the cell division cycle at which the cellular protein would be present at levels high enough to favor the formation of heterodimers. Alternatively, the abil-

ity of EBNA-1 to autoregulate its expression through the region III binding domain may be determined biochemically as the result of posttranslational modification. EBNA-1 does exist in a phosphorylated state (16), which may be functionally distinct from unphosphorylated EBNA-1. Should such posttranslational modification occur within a specific segment of the cell cycle, one would expect to observe alternating periods of high and low Fp activity. In nonsynchronously proliferating cells, therefore, the overall level of activity of Fp observed may be low, as in our experiments. Because EBNA-1-dependent replication of the viral genome is linked to host cell DNA replication (1, 43), it is a reasonable assumption that EBNA-1 is expressed in a cell cycle-dependent manner.

The actual mechanism by which EBNA-1 negatively regulates Fp-directed gene expression is unclear. Our experiments do not distinguish between a transcriptional or posttranscriptional process. Transcriptionally, EBNA-1 could either affect the rate of initiation of transcription or impede progression of the RNA polymerase along the template by binding to DNA. Since the region III binding domain is also present within the RNA transcript, it is conceivable that EBNA-1 acts through an RNA-protein interaction to regulate RNA stability, transport, or processing in a negative manner. Interestingly, the second binding site within domain III spans the junction of the 5′ exon and intron within the primary transcript and, with the exception of the last 3 of 10 nucleotides of this binding site, is conserved in the mature mRNA. This may reflect the potential of EBNA-1 to interact with either the primary transcript or the mRNA.

Although we have addressed the regulation of Fp in a BL cell background, Fp is also active during type II latency in NPC cells (18, 37). In a recently reported analysis of Fp activity in HEp-2 cells employing Fp-CAT reporter plasmids (−959/+24 and −140/+24), Fp activity was found to be low in these epithelial cells (37). This is consistent with our findings. However, since the possible role of downstream elements was not examined in that study, it remains to be determined whether the regulatory elements that we have described are also functional in an epithelial-cell background.

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